

Adipose Tissue: A Vital In Vivo Role in Mammary Gland Development but not Differentiation

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ABSTRACT Development and differentiation of the mammary gland occurs by means of critical stromal-epithelial interactions. Although many studies have attempted to understand these complex interactions, it has been difficult to demonstrate the essential role of adipose tissue in the development and function of the mammary gland. By using the A-ZIP/F-1 transgenic mice lacking in white adipose tissue (WAT), we have studied the role of adipocytes in mammary gland development and differentiation. In the absence of WAT, rudimentary mammary anlagen form but are unable to grow and branch normally, resulting in a few, short, severely distended ducts. However, during pregnancy, a tremendous amount of epithelial cell division and alveolar cell formation occurs even in the absence of adipocytes, illustrating that adipose tissue is not required for mammary gland differentiation. Mammary gland transplantation revealed that epithelial cells from these transgenic mice possess the potential for normal growth and differentiation when placed into a normal stromal environment. These experiments clearly demonstrate that the absence of adipocytes in the mammary gland results in disruption of stromal-epithelial interactions that prevent normal mammary gland development. The rudimentary epithelial anlage, however, contain mammary stem cells, which are fully capable of alveolar differentiation. Published 2002 Wiley-Liss, Inc.†

Key words: adipocytes; mammary gland; development; transgenic; mice

INTRODUCTION

Development of the mammary gland occurs in discrete stages by means of mutual and reciprocal mesenchymal-stromal interactions. To date, our understanding of specific interactions that occur between the epithelial cells and adipocytes, fibroblasts, and other stromal components remains limited. This lack of knowledge is especially notable in the role of the mammary fat pad, which may interact with developing epithelium by means of cellular signaling mechanisms

and/or as a physical element through which epithelial ducts may grow. At 12 days post coitum (dpc) in the mouse, epithelial mammary gland buds are induced in ventral epidermis by mammary mesenchyme (Sakakura et al., 1982). By 16 dpc, lipid has begun to be deposited in mesenchymal cells of the fat pad precursor (Sakakura et al., 1982) followed by ductal branching morphogenesis beginning at day 17 of gestation through inductive effects of the mesenchyme surrounding the rudimentary mammary glands. By 18 dpc, the primary duct has given rise to 3–4 branches, and by birth, 10–15 ductal branches have developed (Sakakura, 1987; reviewed by Cunha, 1994; Cunha and Horn, 1996). From birth, until the initiation of puberty at 3 weeks of age, the mouse mammary gland exhibits only minimal ductal outgrowth. With the onset of puberty, however, a combination of essential systemic hormones (e.g., estrogen) and paracrine hormones (e.g., insulin-like growth factor I, IGF-I) induce terminal end buds (TEBs) to reappear at the ductal tips accompanied by a significant increase in the growth rate. TEBs, stimulated by this combination of hormones (Kleinberg et al., 1985; Walden et al., 1998), drive ductal morphogenesis in the gland by producing a supply of differentiated ductal and myoepithelial cells, resulting in the elongation of the ducts (reviewed by Daniel and Silberstein, 1987). Ductal branching and pattern formation are also determined by growth patterns of the TEBs, which are influenced by interactions with the fat pad.

Further glandular development occurs once sexual maturity has been reached. First, additional ductal side branching and alveolar bud formation occur during each estrus cycle. Second, with the onset of preg-

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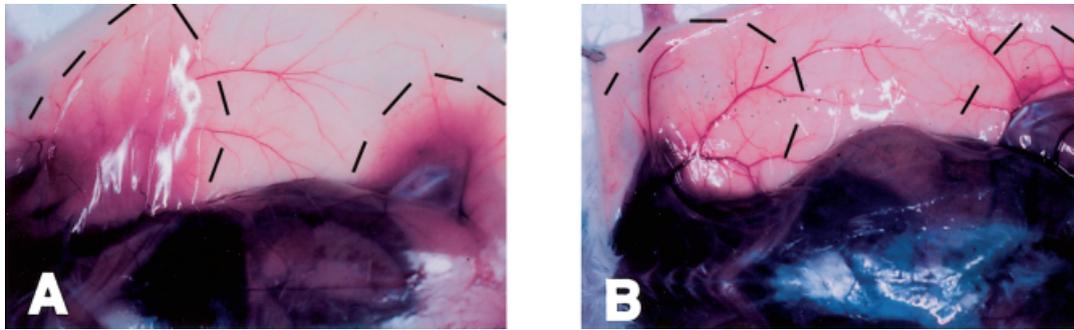


Fig. 1. Adult mammary gland dissection. **A:** Wild-type animals display obvious mammary gland fat pads (surrounded by broken line). **B:** Fat pads are notably absent in A-ZIP/F-1 mice (broken lines indicates where fat pad is expected) when skin is peeled from the body.

nancy and increases in estrogen, progesterone, and prolactin levels, alveolar buds are stimulated to give rise to lobuloalveolar structures capable of milk production. The ability of adult mammary epithelium to grow, undergo ductal morphogenesis, form alveoli, and produce milk proteins has also been shown to be dependent on the connective tissue environment (reviewed by Cunha, 1994). After pregnancy and estrus, the gland undergoes involution with loss of most, but not all, of the epithelial components gained during the preceding event.

Each stage of mammary gland development and differentiation has distinct patterns of gene expression and specific hormone requirements that influence the cross-talk between epithelium and mesenchyme/stroma to regulate development and differentiation (Daniel and Silberstein, 1987; Sakakura, 1987). However, individual stromal-epithelial interactions have been difficult to characterize because *in vitro* studies are unable to closely mimic these complex interactions; monolayer culture conditions and the absence of the varied cell types normally present in the gland result in artificial and sometimes limited cell-cell interactions. *In vivo* models that lack a single genetic or cellular component of the mammary gland provide a more relevant system in which to study stromal-epithelial interactions. Here, we describe how the absence of fat in a transgenic mouse model regulates ductal growth and morphology but not differentiation of the mammary epithelium.

RESULTS

Mammary Gland Fat Pad Is Required for Normal Mammary Ductal Morphogenesis

No mammary fat pad was present in adult A-ZIP/F-1 mice (Fig. 1B) as can clearly be discerned in wild-type mice (Fig. 1A). Whole-mount (Fig. 2A,B) and histologic analysis (Fig. 2C,D) showed that mammary glands were severely hypoplastic with small clusters of ducts in a loose fibrous stroma (Fig. 2C). The stromal component surrounding the ducts

was quite pronounced compared with that of wild-type ducts and demonstrated increased amounts of collagen and fibroblasts. There was some suggestion of increased myoepithelial proliferation in the immediate region around the branching duct. The epithelium itself was often seen to be multilayered and several mice exhibited ductal ectasia with significant dilation of large ducts containing homogenous eosinophilic material (Fig. 2C). Some of these ducts had short stubby ducts extending from them, somewhat resembling the primitive lobule 1 structures as described by (Russo et al., 1990). However, no alveolar buds were identified in the glands analyzed. Fat was absent in all glands examined except for a small cluster of adipocytes associated with the mammary gland in one animal. Mast cells were found to be abundant in the stroma of the transgenic mice.

PAS staining (not shown), and immunostaining for smooth muscle actin, a marker for myoepithelial cells, revealed no differences between the transgenic and wild-type virgin mammary glands (Fig. 2E,F). Figure 2 shows the presence of smooth muscle actin in the differentiated myoepithelial cells surrounding the ductal cells as expected.

Differences in ultrastructures between mammary glands from transgenic and wild-type mice were revealed by electron microscopy. In transgenic mice, the epithelial cells exhibited altered morphologic characteristics with pleomorphic nuclei and increased cytoplasm compared with wild-type mice (Fig. 3). Stromal cells appeared mesenchymal, but intracytoplasmic fibrils were not prominent.

Proliferation and Lobuloalveolar Development Occur in the Absence of Mammary Adipose Tissue

Lobuloalveolar development takes place during pregnancy to prepare the mammary gland for lactation. During this process, terminal alveolar units differentiate from presumptive stem cells. Hormonal requirements for lobuloalveolar development are different

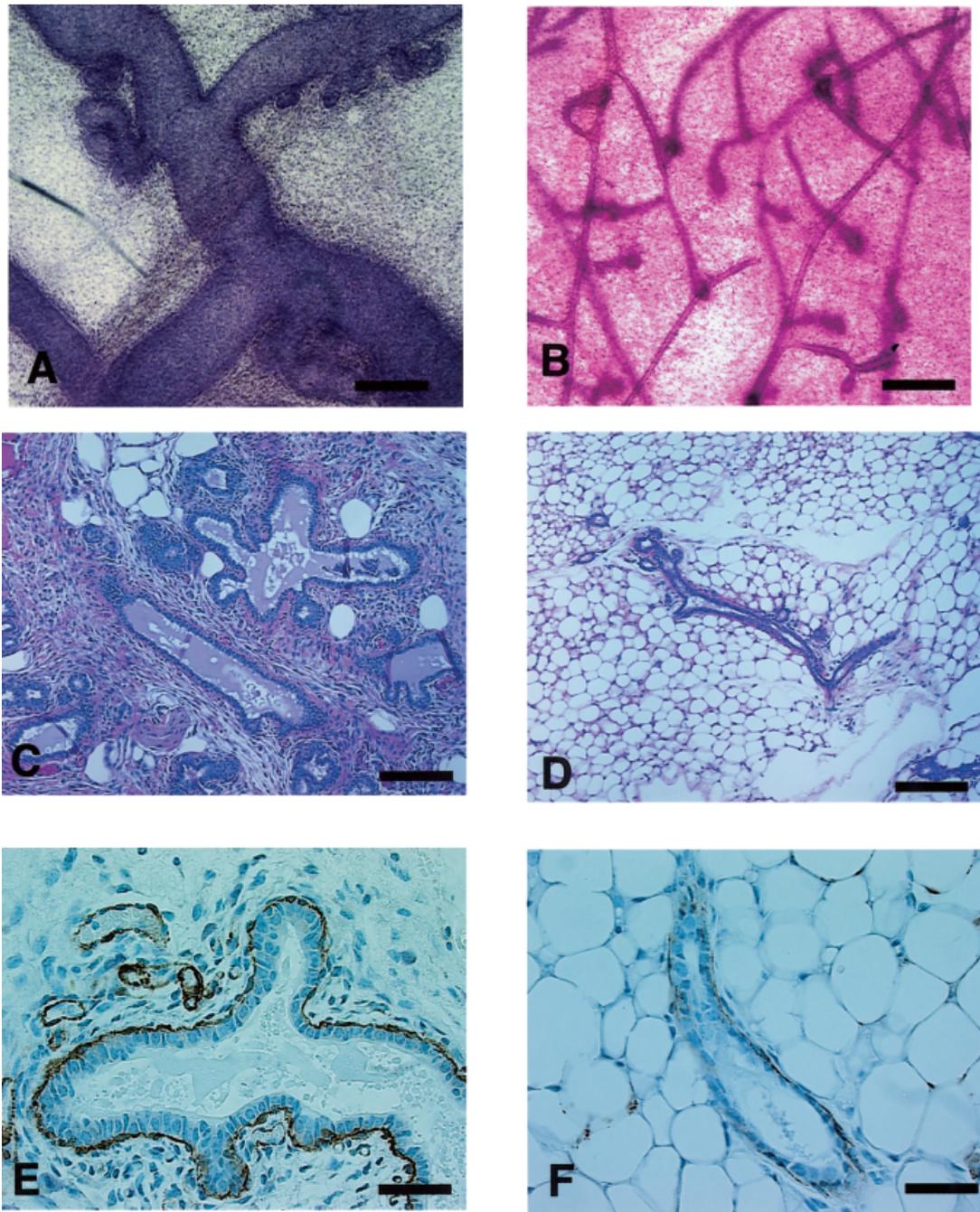


Fig. 2. Whole-mount analysis of mammary glands from 3-month-old A-ZIP/F-1 mice (A) compared with FVB/N mice (B) show only rudimentary ductal formation and ducts are severely distended. Histologic analysis of adult A-ZIP/F-1 mammary glands (C) and age matched FVB/N mammary glands (D). Immunohistochemistry for smooth muscle actin

reveals a basal layer of myoepithelial cells in both A-ZIP/F-1 (E) and wild-type mammary glands (F), suggesting normal structural organization of the rudimentary glands. Scale bars = 500 μm in A,B, 250 μm in C,D, 125 μm in E,F.

from those required for ductal growth; therefore, it is expected that different stromal–epithelial interactions would also take place during this time.

Despite the inability of heterozygous A-ZIP/F-1 females to be able to carry pups to term (Moitra et al.,

1998), it was possible to examine mammary glands in pregnant females. Examination of mammary glands at approximately day 16 of pregnancy showed a marked increase in mammary gland size. From the rudimentary ductal structures present in virgin A-ZIP/F-1

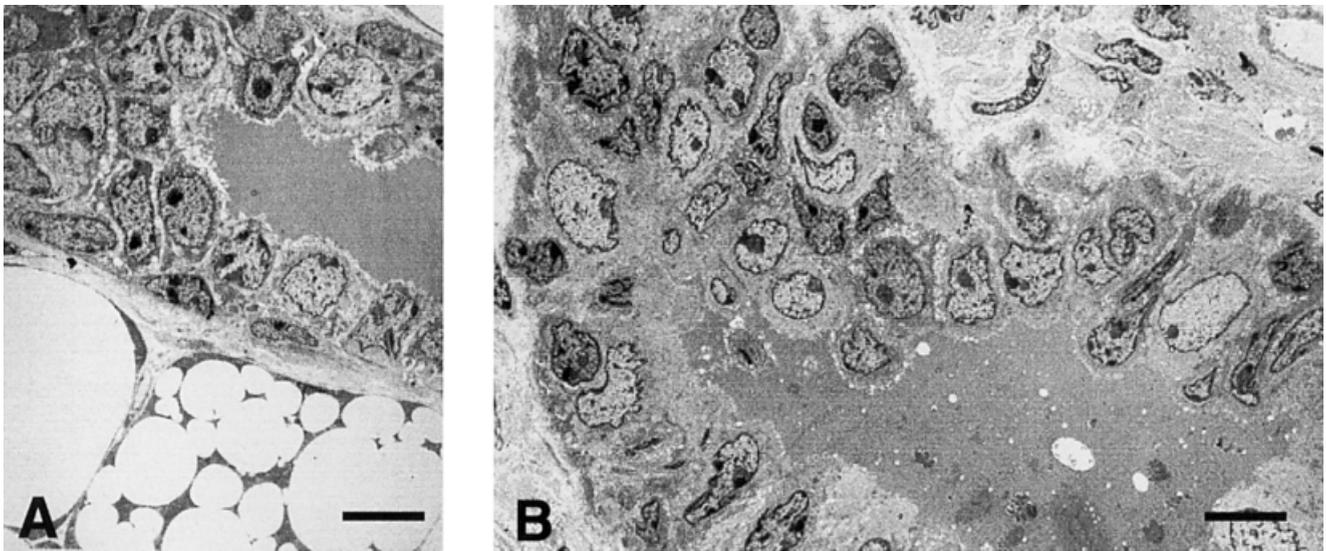


Fig. 3. Electron photomicrographs of mammary ducts in 3-month-old wild-type (A) and A-ZIP/F-1 (B) females showing thickened hyperplastic epithelium. Note the pleomorphic cell nuclei and prominent stromal cells around the duct. Uranyl acetate, lead citrate staining. Scale bars = 10 μ m in A,B.

mice, the inguinal mammary glands had increased in size to approximately 20×7 mm. Whole-mount analyses of these glands revealed extensive lobular proliferation without septation between adipose tissue (Fig. 4A) as is seen in glands of normal pregnant mice (Fig. 4B). Analysis of these differentiated glands (Fig. 4) showed continued distention of the ductal system filled with flocculent eosinophilic material and an absence of fat (Fig. 4C) compared with normal pregnant glands (Fig. 4D). The A-Zip/F1 ducts were surrounded by large numbers of closely apposed alveoli containing large secretory droplets, as normally seen in pregnancy. Smaller ducts and terminal ductules of the type seen in pregnant wild-type mice were not obvious, suggesting that a normal ductal architecture did not develop in association with the alveolar cell proliferation and differentiation. Several disorganized alveolar clusters undergoing lactational type changes were occasionally observed within the lumen of large ducts. Smooth muscle actin staining demonstrated that the alveolar cells in A-ZIP/F-1 mice were surrounded by a layer of myoepithelial cells (Fig. 4E) as is seen in alveolar structures of normal pregnant mice (Fig. 4F). PAS staining for glycoproteins demonstrated abundant collagen and basement membrane components associated with the increased stromal component (Fig. 4G) compared with the wild-type pregnant glands (Fig. 4H). The physical changes seen in alveoli that suggest the development of lactational capability were supported by immunohistochemistry for β -casein. Expression of β -casein (a marker for lactation) was not observed in virgin FVB mammary glands (Fig. 5A) but was seen in both A-ZIP/F-1 mammary glands (Fig. 5B) and pregnant wild-type

FVB mammary glands (Fig. 5C). Further studies will be required to determine how these secretory structural units are connected to the primitive ductal system.

Mammary Epithelial Cells From White Adipose Tissue-Deficient Mice Are Able to Undergo Normal Development

Normal mammary epithelium contains stem cells that are competent to form ducts and secretory alveoli when transplanted into a cleared fat pad (DeOme et al., 1959). Transplantation of fragments from the rudimentary mammary glands found in A-ZIP/F-1 mice into cleared fat pads of 3-week-old FVB/N mice resulted in the growth of ductal structures that completely filled the fat pad (Fig. 6A, C) 8 weeks after transplantation. This ductal growth was indistinguishable from growth and development of wild-type transplants into the same mouse (Fig. 6B,D).

After whole-mount analysis, DNA was extracted from a subset of mammary gland transplants and analyzed for the presence of the transgene by polymerase chain reaction (PCR) and slot blot. As expected, the transgene was detected in A-ZIP/F-1 but not wild-type transplants (data not shown). Thus, epithelial growth in these glands had arisen from the implanted transgenic tissue rather than from contamination of wild-type epithelium. Furthermore, the site of implantation was seen in several fat pads, with ducts extending out from this point.

In a second experiment, glands were transplanted from A-ZIP/F-1 mice into cleared fat pads of 3-week-old FVB/N mice, which were subsequently mated. At parturition, these glands also demonstrated normal devel-

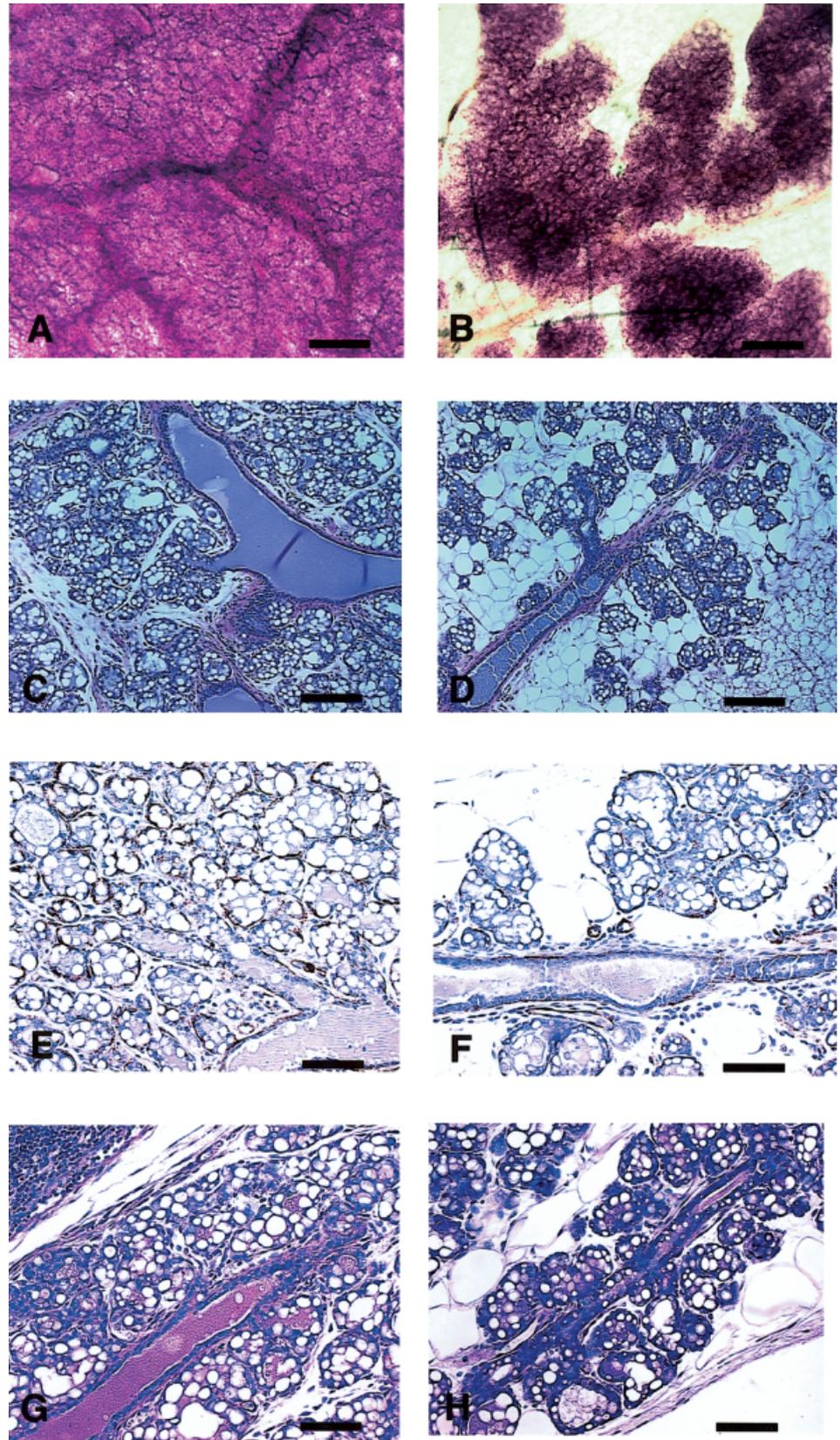


Fig. 4. During pregnancy, extensive epithelial cell proliferation is seen by using whole-mount analysis in mammary glands from wild-type mice (B) as well as in the absence of mammary gland adipocytes in A-ZIP/F-1 mice (A). Histologic analysis of mammary glands from A-ZIP/F-1 mice at day 16 of pregnancy reveals that, in the absence of adipose tissue, there is significant epithelial proliferation with abundant alveolar cells (C) similar to those seen in wild-type mice (D). Immunohistochemistry for smooth muscle actin again reveals basal layers of myoepithelial cells in both A-ZIP/F-1 (E) and wild-type (F) glands. Similarly, PAS staining shows no significant differences between the two genotypes (A-ZIP/F-1 [G], wild-type [H]). Scale bars = 500 μ m in A,B, 250 μ m in C,D, 125 μ m in E-H.

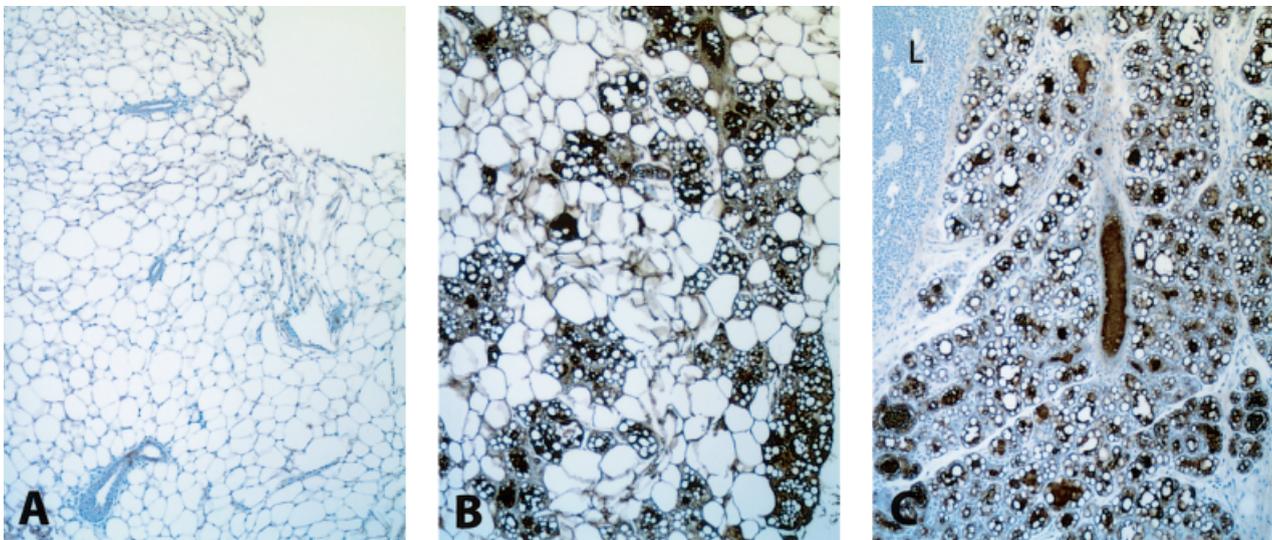


Fig. 5. β -Casein immunohistochemistry. **A:** β -Casein, a marker of lactation, is not expressed in virgin mammary glands as expected. **B,C:** In contrast, mammary glands from pregnant FVB and A-ZIP/F1 transgenic mice show β -casein expression in all alveoli. L, lymph node.

opment and differentiation with full ductal penetration of the fat pad and extensive lobuloalveolar formation (Fig. 6E,G), which was indistinguishable from wild-type transplants by using both whole-mount and histologic analysis (Fig. 6F,H). RNA was isolated from mammary gland transplants at parturition and examined for the expression of milk protein markers (whey acidic protein [WAP] and β -casein) by Northern analysis. All transplants expressed detectable levels of the markers (Fig. 7, lanes A–F). As expected, expression of milk proteins was not detected in RNA isolated from virgin mammary glands (Fig. 7, lane G). These results correspond to expression of β -casein as assessed by immunohistochemistry (Fig. 5).

Steroid Hormone Levels

Along with a variety of hormones, estrogen plays a pivotal role in the development and differentiation of the mammary gland. Lack of estrogen responsiveness in the mammary gland results in only rudimentary ductal formation (Korach et al., 1996). Serum estradiol levels in A-ZIP/F-1 mice were, therefore, measured to ensure that the hypoplastic mammary gland phenotype observed was not simply due to abnormally low estradiol levels. Serum estradiol levels in transgenic mice were the same as those measured in wild-type mice, i.e., 12.9 pg/ml (SEM 2.7, $n = 6$), 15.9 pg/ml (SEM 1.5, $n = 8$), respectively.

DISCUSSION

Mammary Gland Development and Differentiation in the Absence of Adipose Tissue

The rudimentary ductal system found in A-ZIP/F-1 transgenic mice is the first in vivo demonstration that

adipocytes are vital for the ductal development in the mammary gland. This finding is in agreement with previous in vitro studies showing that preadipocytes stimulate epithelial growth (Levine and Stockdale, 1984; Wiens et al., 1987). The rudimentary ductal structure observed in A-ZIP/F-1 mice is similar to the previously described phenotype described for mice lacking $ER\alpha$ (Korach et al., 1996), although ducts from A-ZIP/F-1 females also showed severe distension. However, given that estradiol levels were normal and females were able to ovulate, there was no evidence to suggest that the estrogen signaling pathway had been disrupted in this mouse model. The presence of only three to four ducts in adult A-ZIP/F-1 females suggests that ductal growth and branching had ceased at around 18 dpc in these mammary glands. This period of embryonic development corresponds to the time shortly after adipocytes have begun to accumulate in the region of the developing ductal system (Sakakura, 1987) and it is possible that lack of adipocyte differentiation causes stunted ductal growth. It is unclear whether the essential white adipose tissue (WAT)–epithelial interactions are paracrine interactions of growth factors/steroid hormones, physical interactions, or a combination of both.

In addition to the apparent arrest of ductal branching at around 18 dpc, the reappearance of TEBs at 3 weeks of age that is normally seen in mice does not seem to occur in A-ZIP/F-1 females. The rise in serum estrogen at 3 weeks of age stimulates puberal ductal growth in combination with a variety of hormones, including prolactin, growth hormone (GH), and adrenocorticoids (Gardner and White, 1941; Lyons et al., 1958; Nandi, 1958; reviewed by Daniel and Silberstein,

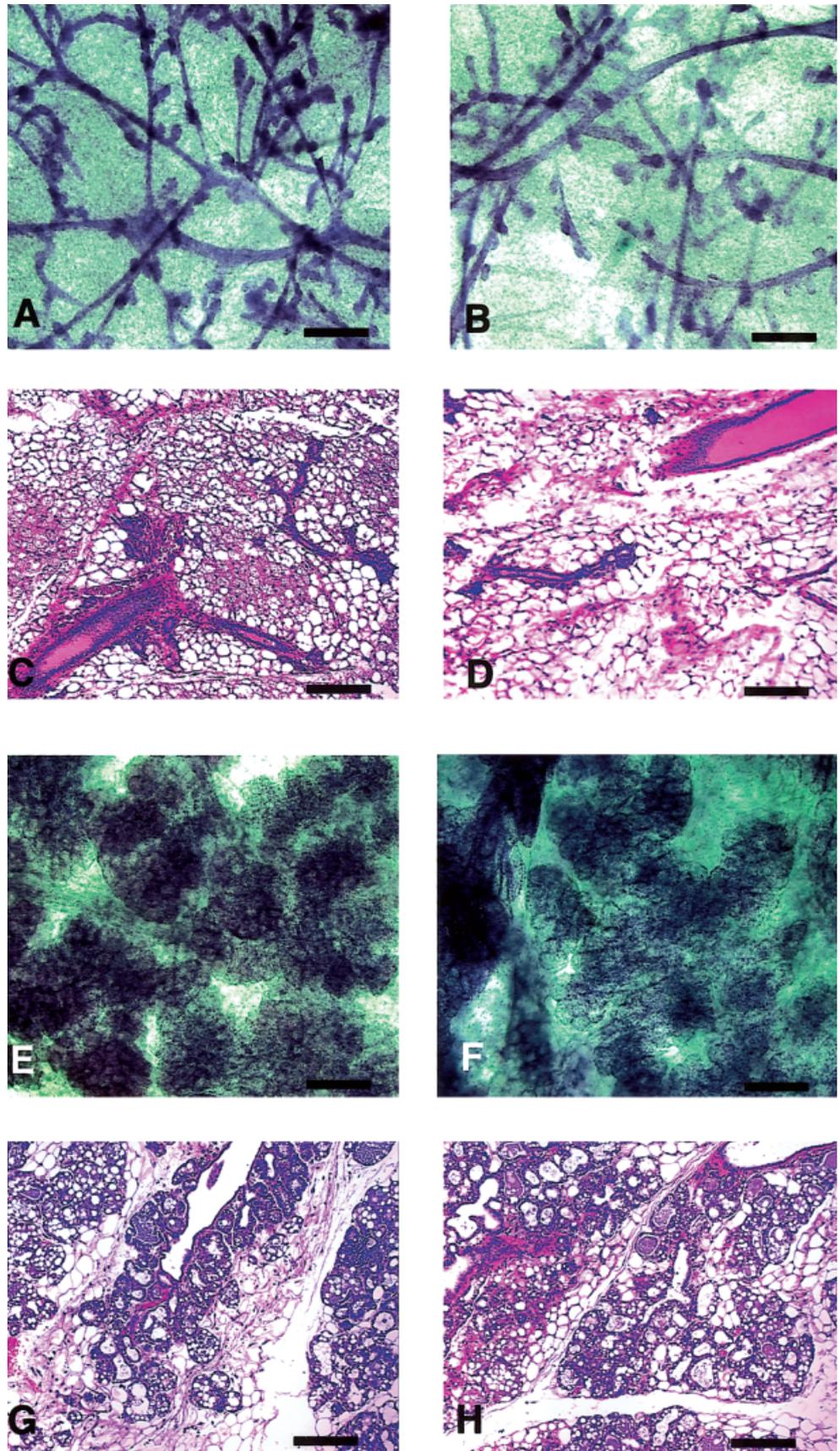


Fig. 6. When transplanted into a normal stromal environment, the rudimentary distended mammary gland ducts from A-ZIP/F-1 mice are able to generate a mammary gland with normal morphologic structure, which fills the entire fat pad (A); mammary gland transplants from wild-type mice into the contralateral inguinal gland of the same mouse (B). No differences were detected in histologic sections comparing A-ZIP/F-1 transgenic (C,E) and wild-type (D,F) transplants (virgin C, D, and lactating E-H). Scale bars = 500 μ m in A,D,E,F, 250 μ m in B,C,G,H.

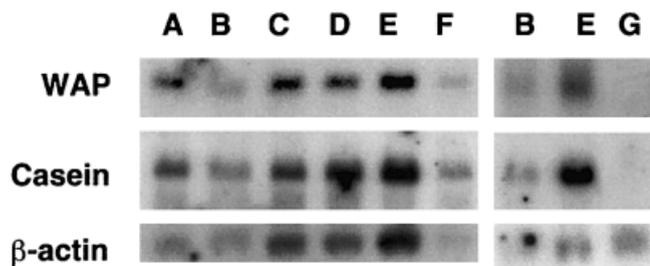


Fig. 7. Northern analysis of transplanted mammary glands from mice 1 day postpartum. **A,C,E**: Transplants from wild-type females. **B,D,F**: Transplants from A-ZIP/F1 mice. Lane G contains RNA from a virgin mammary gland. All glands from lactating animals express the lactation markers WAP and casein, whereas the virgin gland does not.

1987; Kleinberg et al., 2000). There is much evidence supporting the notion that, in addition to hormones exerting their action directly on epithelial cells, they also signal to the epithelium through the stromal component of the mammary gland. One example of this finding is the induction of mammary stroma to produce IGF-I by GH (Kleinberg et al., 1985; Walden et al., 1998). In fact, no other hormones have been shown to stimulate formation of the vital TEBs unless GH or IGF-I is present. Therefore, it is possible that a second mechanism (i.e., lack of IGF-I production) is contributing to the mammary phenotype in the A-ZIP/F-1 transgenics. TEBs are the major sites of cell proliferation for ductal elongation, thus, the absence of TEBs would prevent normal puberal elongation. Given that TEBs disappear when they reach the edge of the mammary fat pad, failure of the TEBs to reappear at puberty may also be due, at least in part, to the physical absence of a fat pad. However, it is not clear whether this is the result of a physical or hormonal interaction.

The reason for ductal distension is not yet clear. It is possible that this distension is simply a secondary phenotype that occurs when normal branching and growth are blocked and secretions from ductal cells build up within the ducts.

In contrast to the lack of ductal development, alveolar development occurred in A-ZIP/F-1 females during pregnancy to produce a mass of lobuloalveolar structures. The ability of epithelial cells to undergo proliferation, lobuloalveolar formation and show β -casein expression during pregnancy, even in the absence of adipose tissue, demonstrates that interactions between mammary epithelial cells and adipocytes are not essential in this process. This phenotype, the lack of ductal growth, but potential for alveolar formation, is similar to that reported for epidermal growth factor receptor (EGFR) knockout mice (Wiesen et al., 1999). Studies performed on EGFR knockout mice have demonstrated that signaling through EGFR is not essential for alveolar formation, even though it is indispensable for ductal development. It could be suggested that in A-ZIP/F-1 mice, it is simply the lack of stromal EGFR

(through the absence of a mammary fat pad) that gives rise to this phenotype. However, EGFR is expressed in the stromal cells surrounding the terminal end buds, cap cells of end buds, myoepithelial cells, and luminal epithelial cells, as well as in adipocytes (Coleman et al., 1988; Coleman and Daniel, 1990; DiAugustine et al., 1997). Therefore, at least some EGFR is expected to be present in A-ZIP/F-1 mammary glands. Considered together with previously described results (Lydon et al., 1995; Ormandy et al., 1997), these models support the hypothesis that, although ductal and alveolar development in the mammary gland share key regulatory molecules that transduce systemic hormone action, ductal and alveolar development are also controlled by distinct pathways at the local level by growth factors and hormones by means of different stromal-epithelial interactions.

MATERIALS AND METHODS

Mice and Determination of Genotypes

A-ZIP/F-1 transgenic mice lacking WAT, previously described by (Moitra et al., 1998), were maintained by breeding heterozygous males with FVB/N females. All animals studied were either heterozygous for the transgene or wild-type. Mice were maintained in compliance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Research Council. Genotyping was performed by PCR amplification of DNA extracted from tail biopsies and tissue implants (primers 5'ctgtgctgcagaccaccatgg, 3'ccgcgaggtcgtccagcctca). Amplification conditions were: initial denaturation 95°C followed by 30 cycles of 94°C 1 min, 61°C 1 min, 72°C 1 min, 72°C 10 min. Slot blot analysis was performed by using standard techniques. The FLAG sequence of the transgene was radioactively labeled and used as a probe.

Mammary Gland Dissection and Transplantation

Mice were euthanized with carbon dioxide. Inguinal glands (number 4, 5, 9, and 10 glands) were removed from adult females and spread onto glass slides for whole-mount analysis. After overnight fixation in 70% ethanol, glands were rehydrated, stained with carmine alum, and dehydrated; samples were then cleared in xylene and mounted (Sympson et al., 1994). For paraffin embedding, glands were fixed in freshly prepared 4% paraformaldehyde and subsequently transferred to 70% ethanol for storage until embedding. Sections 5 μ m in thickness were cut and stained with hematoxylin and eosin, periodic acid-Schiff staining (PAS), or used for immunohistochemical analysis (smooth muscle α -actin).

In transplantation studies, endogenous epithelium of the no. 4 and no. 9 (inguinal) glands was removed from 24 three-week-old FVB/N mice as previously described by (DeOme et al., 1959). Under avertin anesthesia, an incision was made in the cleared fat pad, and a small

piece of donor mammary gland (FVB/N into fat pad no. 4 and A-ZIP/F-1 into no. 9). Eight weeks after surgery, half of the mice were killed and mammary glands were analyzed. The remaining mice were mated and the transplanted tissues were collected for whole-mount and histologic analysis (as described above) within 24 hr of delivery.

Immunohistochemistry

Sections (5 μm) were prepared from paraformaldehyde-fixed mammary gland tissues described above. A rabbit polyclonal antibody (provided by G. Smith) was used to detect β -casein expression in these sections.

Electron Microscopy

Tissue preparation for electron microscopy (EM) study is described in detail (Hayat, 1970). Briefly, mammary gland tissue was initially fixed in 2% glutaraldehyde in PBS (phosphate buffer saline, pH 7.4) followed by post-fixation in 1% osmium in sodium cacodylate buffer (0.1 M, pH 7.4). The tissue was washed thoroughly with sodium cacodylate buffer and dehydrated in graded alcohol and propylene oxide. The tissue was infiltrated in an equal mixture of epoxy resin and propylene oxide overnight at room temperature and embedded in the pure resin and cured at 60°C for 48 hr. The cured block was sectioned (0.5 μm) and stained with 0.5% Toluidine Blue-O in sodium borate buffer (1%) for light microscopy to select the area for thin sectioned EM analysis. The selected area was thin sectioned and mounted on a Formvar-film, one-hole grid and stained with uranyl acetate and lead citrate. The thin-sectioned tissue was examined and photographed with an electron microscope operated at 75 kV.

Northern Blot Analysis

Mammary glands from epithelial transplantation were collected; half of the gland was used either for whole-mount or histologic analysis. RNA was isolated from the remaining tissue by using Qiagen RNeasy columns (Qiagen, Inc., Valencia, CA). Northern blot analysis was performed with 10 μg of total RNA by using standard techniques (Sambrook et al., 1989). Membranes were probed with radiolabeled antisense oligonucleotides to WAP (caacgcatggtaccggtgtca) and β -casein (gtctctcttgaagagcaagggcc) as described by L. Henninghausen (<http://mammary.nih.gov/>)

Estradiol Measurements

Blood was collected from the retro-orbital plexus of mice under avertin anesthesia, and serum was collected by centrifugation and stored at -80°C until analysis. Serum estradiol levels were measured by radioimmunoassay by using the DPC kit (Diagnostic Products Corp, Los Angeles, CA) following the manufacturer's instructions.

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